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(71) Applicant (for all designated States except US): DE-GUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).

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(72) Inventor; and

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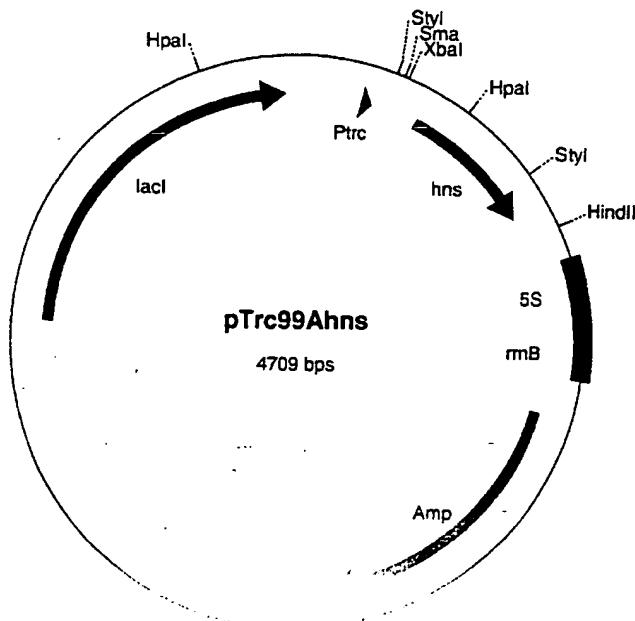
(75) Inventor/Applicant (for US only): RIEPING, Mechthild [DE/DE]; Mönkebergstrasse 1, 33619 Bielefeld (DE).

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(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY



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(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine.

**Process for the Preparation of L-Amino Acids using Strains of the Enterobacteriaceae Family**

**Field of the Invention**

This invention relates to a process for the preparation of 5 L-amino acids, in particular L-threonine, using strains of the Enterobacteriaceae family in which at least the *hns* gene is enhanced.

**Prior Art**

L-Amino acids, in particular L-threonine, are used in human 10 medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

It is known to prepare L-amino acids by fermentation of strains of Enterobacteriaceae, in particular *Escherichia coli* (*E. coli*) and *Serratia marcescens*. Because of their 15 great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as e.g. stirring and supply of oxygen, or the composition of the 20 nutrient media, such as e.g. the sugar concentration during the fermentation, or the working up to the product form, by e.g. ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are 25 used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the threonine analogue  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid (AHV), or are auxotrophic for 30 metabolites of regulatory importance and produce L-amino acid, such as e.g. L-threonine, are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid 5 biosynthesis genes and investigating the effect on the production.

#### Object of the Invention

The object of the invention is to provide new measures for improved fermentative preparation of L-amino acids, in 10 particular L-threonine.

#### Summary of the Invention

The invention provides a process for the preparation of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which in 15 particular already produce L-amino acids and in which the nucleotide sequence which codes for the hns gene is enhanced.

#### Detailed Description of the Invention

Where L-amino acids or amino acids are mentioned in the 20 following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-25 histidine, L-lysine, L-tryptophan and L-arginine. L-Threonine is particularly preferred.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by 30 the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or a gene or allele which codes for a corresponding enzyme or

protein with a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is 5 in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

10 The process is characterized in that the following steps are carried out:

- a) fermentation of microorganisms of the Enterobacteriaceae family in which the hns gene is enhanced,
- 15 b) concentration of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the Enterobacteriaceae family, and
- c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100%) thereof 20 optionally remaining in the product.

The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally 25 cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family chosen from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. Of the genus Escherichia the species Escherichia 30 coli and of the genus Serratia the species Serratia marcescens are to be mentioned in particular.

Suitable strains, which produce L-threonine in particular, of the genus *Escherichia*, in particular of the species *Escherichia coli*, are, for example

Escherichia coli TF427  
5 Escherichia coli H4578  
Escherichia coli KY10935  
Escherichia coli VNIIgenetika MG442  
Escherichia coli VNIIgenetika M1  
Escherichia coli VNIIgenetika 472T23  
10 Escherichia coli BKIIM B-3996  
Escherichia coli kat 13  
Escherichia coli KCCM-10132.

Suitable L-threonine-producing strains of the genus *Serratia*, in particular of the species *Serratia marcescens*,  
15 are, for example

*Serratia marcescens* HNr21  
*Serratia marcescens* TLr156  
*Serratia marcescens* T2000.

Strains from the Enterobacteriaceae family which produce L-threonine preferably have, inter alia, one or more genetic or phenotypic features chosen from the group consisting of: resistance to  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to  $\alpha$ -methylserine, resistance to diaminosuccinic acid,  
25 resistance to  $\alpha$ -aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues, such as, for example, valine hydroxamate, resistance to purine analogues, such as, for example, 6-dimethylaminopurine, a need for L-methionine, optionally a  
30 partial and compensable need for L-isoleucine, a need for meso-diaminopimelic acid, auxotrophy in respect of threonine-containing dipeptides, resistance to L-threonine, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid.

resistance to L-aspartate, resistance to L-leucine, resistance to L-phenylalanine, resistance to L-serine, resistance to L-cysteine, resistance to L-valine, sensitivity to fluoropyruvate, defective threonine

- 5 dehydrogenase, optionally an ability for sucrose utilization, enhancement of the threonine operon, enhancement of homoserine dehydrogenase I-aspartate kinase I, preferably of the feed back resistant form, enhancement of homoserine kinase, enhancement of threonine synthase,
- 10 enhancement of aspartate kinase, optionally of the feed back resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenol pyruvate carboxylase, optionally of the feed back resistant form, enhancement of phosphoenol pyruvate synthase, enhancement
- 15 of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of acetic acid formation.

It has been found that microorganisms of the

- 20 Enterobacteriaceae family produce L-amino acids, in particular L-threonine, in an improved manner after enhancement, in particular over-expression, of the hns gene.

The use of endogenous genes is in general preferred.

- 25 "Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or nucleotide sequences present in the population of a species.

- 30 The nucleotide sequences of the genes of *Escherichia coli* belong to the prior art and can also be found in the genome sequence of *Escherichia coli* published by Blattner et al. (Science 277: 1453-1462 (1997)).

The following information on the hns gene is known, inter alia, from the prior art:

Description: DNA-binding protein HLP-II (= HU, BH2, HD, NS); pleiotropic regulator, histone-like protein HN-S

Reference: Pon et al.; Molecular and General Genetics 212(2): 199-202 (1988) Bertin et al.; Biochimie 83(2): 235-241 (2001) Azam et al.; Journal of Bacteriology 181(20): 6361-6370 (1999)

Accession No.: AE000222

10 Alternative gene names: bglY, cur, drc, drdX, drs, fimG, mysA, msyA, osmZ, pilG, topS, topX, virR, hnsA,

The nucleic acid sequences can be found in the databanks of the National Center for Biotechnology Information (NCBI) of 15 the National Library of Medicine (Bethesda, MD, USA), the nucleotide sequence databank of the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany or Cambridge, UK) or the DNA databank of Japan (DDBJ, Mishima, Japan).

20 Alleles of the hns gene which result from the degeneracy of the genetic code or due to "sense mutations" of neutral function can furthermore be used.

To achieve an enhancement, for example, expression of the genes or the catalytic properties of the proteins can be 25 increased. The two measures can optionally be combined.

To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes 30 which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative L-threonine production. The expression is likewise improved by measures to prolong the

life of the m-RNA. Furthermore, the enzyme activity is also enhanced by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can 5 be integrated and amplified in the chromosome.

Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, 10 inter alia, in Chang and Cohen (Journal of Bacteriology 134: 1141-1156 (1978)), in Hartley and Gregori (Gene 13: 347-353 (1981)), in Amann and Brosius (Gene 40: 183-190 (1985)), in de Broer et al. (Proceedings of the National 15 Academy of Sciences of the United States of America 80: 21-25 (1983)), in LaVallie et al. (BIO/TECHNOLOGY 11: 187-193 (1993)), in PCT/US97/13359, in Llosa et al. (Plasmid 26: 222-224 (1991)), in Quandt and Klipp (Gene 80: 161-169 (1989)), in Hamilton (Journal of Bacteriology 171: 4617-4622 (1989)), in Jensen and Hammer (Biotechnology and 20 Bioengineering 58: 191-195 (1998)) and in known textbooks of genetics and molecular biology.

Plasmid vectors which are capable of replication in Enterobacteriaceae, such as e.g. cloning vectors derived from pACYC184 (Bartolomé et al.; Gene 102: 75-78 (1991)), 25 pTrc99A (Amann et al.; Gene 69: 301-315 (1988)) or pSC101 derivatives (Vocke and Bastia, Proceedings of the National Academy of Sciences USA 80(21): 6557-6561 (1983)) can be used. A strain transformed with a plasmid vector, wherein 30 the plasmid vector carries at least one nucleotide sequence which codes for the hns gene, can be employed in a process according to the invention.

It is also possible to transfer mutations which affect the expression of the particular gene into various strains by sequence exchange (Hamilton et al. (Journal of Bacteriology 35 80(21): 6557-6561 (1983)), and by other means.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family to enhance one or more enzymes of the known threonine biosynthesis pathway or enzymes of 5 anaplerotic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate, in addition to the enhancement of the *hns* gene.

Thus, for example, one or more of the genes chosen from the group consisting of

- 10 • the *thrABC* operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
- the *pyc* gene which codes for pyruvate carboxylase (DE-A-19 831 609),
- 15 • the *pps* gene which codes for phosphoenol pyruvate synthase (Molecular and General Genetics 231(2): 332-336 (1992)),
- the *ppc* gene which codes for phosphoenol pyruvate carboxylase (Gene 31: 279-283 (1984)),
- 20 • the *pntA* and *pntB* genes which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),
- the *rhtB* gene which imparts homoserine resistance (EP-A-0 994 190),
- 25 • the *mgo* gene which codes for malate:quinone oxidoreductase (WO 02/06459),
- the *rhtC* gene which imparts threonine resistance (EP-A-1 013 765),
- the *thrE* gene of *Corynebacterium glutamicum* which codes for the threonine export protein (WO 01/92545),

- the *gdhA* gene which codes for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23: 199-209 (1983)),
- the *dps* gene which codes for the global regulator *Dps* (Genes & Development 6(12B): 2646-2654 (1992), Accession No. AE000183),
- the *lrp* gene which codes for the regulator of the leucine *Lrp* regulon and high-affinity transport systems of branched-chain amino acids (Journal of Biological Chemistry 266(17): 10768-10774 (1991), Accession No. AE000191),
- the *pgm* gene which codes for phosphoglucomutase (Journal of Bacteriology 176: 5847-5851 (1994), Accession No. AE000172),
- the *fba* gene which codes for fructose bisphosphate aldolase (Biochemical Journal 257: 529-534 (1989), Accession No. AE000376),
- the *ptsG* gene which codes for the glucose-specific IIBC component of the phosphotransferase system PTS (Journal of Biological Chemistry 261(35): 16398-16403 (1986), Accession No. AE000210),
- the *ptsH* gene of the *ptsHIcrr* operon which codes for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),
- the *ptsI* gene of the *ptsHIcrr* operon which codes for enzyme I of the phosphotransferase system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),
- the *crr* gene of the *ptsHIcrr* operon which codes for the glucose-6-phosphate IIIC component of the phosphotransferase system PTS

system PTS (Journal of Biological Chemistry 262(33): 16241-16253 (1987), Accession No. AE000329),

- the *mopB* gene which codes for chaperone GroES (Journal of Biological Chemistry 261(26): 12414-12419 (1986),  
5 Accession No. AE000487),
- the *ahpC* gene of the *ahpCF* operon which codes for the small subunit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92(17): 7617-7621 (1995), Accession No. AE000166), and
- 10 • the *ahpF* gene of the *ahpCF* operon which codes for the large subunit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92(17): 7617-7621 (1995), Accession No. AE000166),

can be enhanced, in particular over-expressed.

15 The use of endogenous genes is in general preferred.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to the enhancement of the *hns* gene, for one or more of the genes chosen from the group consisting of

- 20 • the *tdh* gene which codes for threonine dehydrogenase (Journal of Bacteriology 169: 4716-4721 (1987)),
- the *mdh* gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Archives in Microbiology 149: 36-42 (1987)),
- the gene product of the open reading frame (orf) *yjfA*  
25 (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the gene product of the open reading frame (orf) *ytfP* (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),

- the *pckA* gene which codes for the enzyme phosphoenol pyruvate carboxykinase (Journal of Bacteriology 172: 7151-7156 (1990)),
- the *poxB* gene which codes for pyruvate oxidase (Nucleic 5 Acids Research 14(13): 5449-5460 (1986)),
- the *aceA* gene which codes for the enzyme isocitrate lyase (Journal of Bacteriology 170: 4528-4536 (1988)),
- the *dgsA* gene which codes for the *DgsA* regulator of the phosphotransferase system (Bioscience, Biotechnology and 10 Biochemistry 59: 256-251 (1995)) and is also known under the name of the *mlc* gene,
- the *fruR* gene which codes for the fructose repressor (Molecular and General Genetics 226: 332-336 (1991)) and is also known under the name of the *cra* gene, and
- 15 • the *rpoS* gene which codes for the  $\sigma^{38}$  factor (WO 01/05939) and is also known under the name of the *katF* gene,

to be attenuated, in particular eliminated or for the expression thereof to be reduced.

20 The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or a gene or allele which codes for a 25 corresponding enzyme with a low activity or inactivates the corresponding enzyme (protein) or gene, and optionally combining these measures.

By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 30 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of

the activity or concentration of the protein in the starting microorganism.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to the 5 enhancement of the hns gene, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

- 10 The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed batch (feed process) or the repeated fed batch process (repetitive feed process). A summary of known culture methods is described in the textbook by Chmiel
- 15 (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral 20 Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General 25 Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower 30 oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source

of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

10 Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate,

15 which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to

20 the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed

25 in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of

30 plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25°C to 45°C, and preferably 30°C to 40°C. Culturing is continued until a maximum of L-amino

acids or L-threonine has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry 30: 1190-1206 (1958), or it can take place by reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

The process according to the invention is used for the fermentative preparation of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine, in particular L-threonine.

The present invention is explained in more detail in the following with the aid of embodiment examples.

15 The minimal (M9) and complete media (LB) for *Escherichia coli* used are described by J.H. Miller (A Short Course in Bacterial Genetics (1992), Cold Spring Harbor Laboratory Press). The isolation of plasmid DNA from *Escherichia coli* and all techniques of restriction, ligation, Klenow and  
20 alkaline phosphatase treatment are carried out by the method of Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). Unless described otherwise, the transformation of *Escherichia coli* is carried out by the method of Chung et al. (Proceedings  
25 of the National Academy of Sciences of the United States of America (1989) 86: 2172-2175).

The incubation temperature for the preparation of strains and transformants is 37°C.

### Example 1

### 30 Construction of the expression plasmid pTrc99Ahns

The *hns* gene from *E. coli* K12 is amplified using the

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oligonucleotides. Starting from the nucleotide sequence of the hns gene in *E. coli* K12 MG1655 (Accession Number AE000222, Blattner et al. (Science 277: 1453 - 1462 (1997)), PCR primers are synthesized (MWG Biotech, 5 Ebersberg, Germany):

hns1: 5' - GTTGAGATTACTACAATGAGCG - 3' (SEQ ID No. 1)

hns2: 5' - GCAAGTGCAATCTACAAAAG - 3' (SEQ ID No. 2)

The chromosomal *E. coli* K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions 10 with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 450 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase 15 (Promega Corporation, Madison, USA). The PCR product is ligated with the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) in accordance with the manufacturer's instructions and transformed in the *E. coli* strain TOP10. Selection for 20 plasmid-carrying cells is made on LB agar, to which 50 µg/ml kanamycin are added. After isolation of the plasmid DNA the vector pCR-Blunt II-TOPO-hns is cleaved with the restriction enzymes HindIII and XbaI and, after separation in 0.8% agarose gel with the aid of the QIAquick 25 Gel Extraction Kit (QIAGEN, Hilden, Germany), the hns fragment is isolated. The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI and ligated with the hns fragment isolated. The *E. coli* strain XL1-Blue MRF' (Stratagene, La 30 Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes HpaI, SmaI and StyI. The plasmid 35 is called pTrc99A-hns (Figure 1).

Example 2

## Preparation of L-threonine with the strain MG442/pTrc99Ahns

The L-threonine-producing *E. coli* strain MG442 is described in the patent specification US-A- 4,278,765 and deposited 5 as CMTM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

The strain MG442 is transformed with the expression plasmid pTrc99Ahns described in example 1 and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar 10 with 50 µg/ml ampicillin. The strains MG442/pTrc99Ahns and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{NH}_4\text{Cl}$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/l 15 glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l 20  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland). 250 µl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l 25  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot 1\text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, 30 but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 606 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction 5 with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	L-Threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99Ahns	4.2	2.4

Brief Description of the Figure:

10 • Figure 1: Map of the plasmid pTrc99Ahns containing the hns gene.

The length data are to be understood as approx. data. The abbreviations and designations used have the following meaning:

15 • Amp: Ampicillin resistance gene

• lacI: Gene for the repressor protein of the trc promoter

• P<sub>trc</sub>: trc promoter region, IPTG-inducible

• hns: Coding region of the hns gene

20 • 5S: 5S rRNA region

• 16S: 16S rRNA region

The abbreviations for the restriction enzymes have the following meaning

- HindIII: Restriction endonuclease from *Haemophilus influenzae*
- 5   • HpaI: Restriction endonuclease from *Haemophilus parainfluenzae*
- SmaI: Restriction endonuclease from *Serratia marcescens*
- StyI: Restriction endonuclease from *Salmonella typhi*
- XbaI: Restriction endonuclease from *Xanthomonas campestris*

10

**What is claimed is:**

1. Process for the preparation of L-amino acids, in particular L-threonine, wherein the following steps are carried out:
  - 5 a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which the hns gene or the nucleotide sequence which codes for this, is enhanced, in particular over-expressed,
  - 10 b) concentration of the desired L-amino acid in the medium or in the cells of the microorganisms, and
  - c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100%) thereof
- 15 2. Process according to claim 1, wherein microorganisms in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.
- 20 3. Process according to claim 1, wherein microorganisms in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
- 25 4. Process according to claim 1, wherein the expression of the polynucleotide which codes for the hns gene is increased.
- 30 5. Process according to claim 1, wherein the regulatory and/or catalytic properties of the polypeptide (protein) for which the polynucleotide hns codes are improved or increased.

6. Process according to claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the 5 group consisting of:

6.1 the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,

10 6.2 the pyc gene which codes for pyruvate carboxylase,

6.3 the pps gene which codes for phosphoenol pyruvate synthase,

6.4 the ppc gene which codes for phosphoenol pyruvate carboxylase,

15 6.5 the pntA and pntB genes which code for transhydrogenase,

6.6 the rhtB gene which imparts homoserine resistance,

20 6.7 the mqo gene which codes for malate:quinone oxidoreductase,

6.8 the rhtC gene which imparts threonine resistance,

6.9 the thrE gene which codes for the threonine export protein,

25 6.10 the gdhA gene which codes for glutamate dehydrogenase,

6.11 the dps gene which codes for the global regulator Dps,

6.12 the *lrp* gene which codes for the regulator of the leucine *Lrp* regulon,

6.13 the *pgm* gene which codes for phosphoglucomutase,

5 6.14 the *fba* gene which codes for fructose bisphosphate aldolase,

6.15 the *ptSG* gene which codes for the glucose-specific IIBC component,

10 6.16 the *ptsH* gene which codes for the phosphohistidine protein hexose phosphotransferase,

6.17 the *ptsI* gene which codes for enzyme I of the phosphotransferase system,

15 6.18 the *crr* gene which codes for the glucose-specific IIA component,

6.19 the *mopB* gene which codes for chaperone GroES,

6.20 the *ahpC* gene which codes for the small sub-unit of alkyl hydroperoxide reductase,

20 6.21 the *ahpF* gene which codes for the large sub-unit of alkyl hydroperoxide reductase,

is or are enhanced, in particular over-expressed, are fermented.

7. Process according to claim 1, wherein, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which in addition at the same time one or more of the genes chosen from the group consisting of:

25 7.1 the *tdh* gene which codes for threonine dehydrogenase,

7.2 the *mdh* gene which codes for malate dehydrogenase,

7.3 the gene product of the open reading frame (orf) *yjfA*,

5 7.4 the gene product of the open reading frame (orf) *ytfP*,

7.5 the *pckA* gene which codes for phosphoenol pyruvate carboxykinase,

7.6 the *poxB* gene which codes for pyruvate oxidase,

10 7.7 the *aceA* gene which codes for isocitrate lyase,

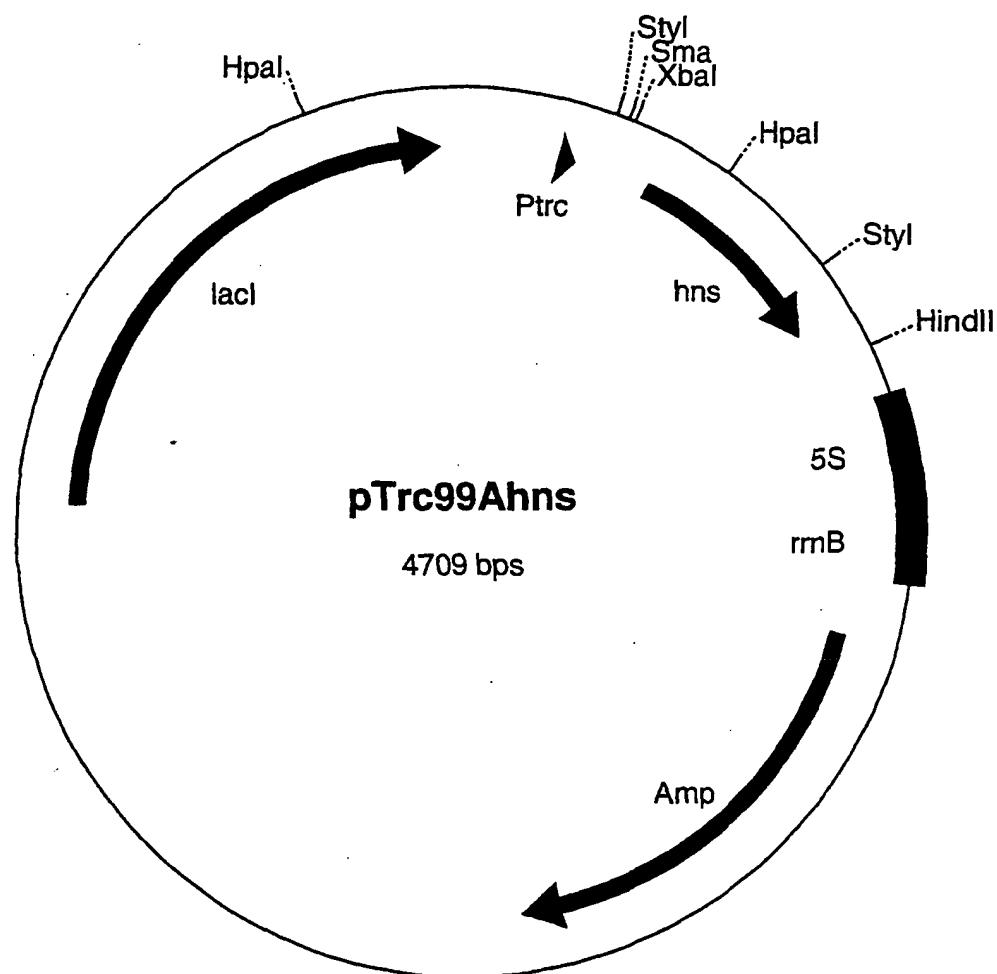
7.8 the *dgsA* gene which codes for the *DgsA* regulator of the phosphotransferase system,

7.9 the *fruR* gene which codes for the fructose repressor, and

15 7.10 the *rpoS* gene which codes for the  $\sigma^{38}$  factor

is or are attenuated, in particular eliminated or reduced in expression, are fermented.

Figure 1:



## SEQUENCE PROTOCOL

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strains of the Enterobacteriaceae family  
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<170> PatentIn version 3.1  
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40

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LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,  
SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,  
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European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,  
GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent  
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Published:

— with international search report

(72) Inventor; and

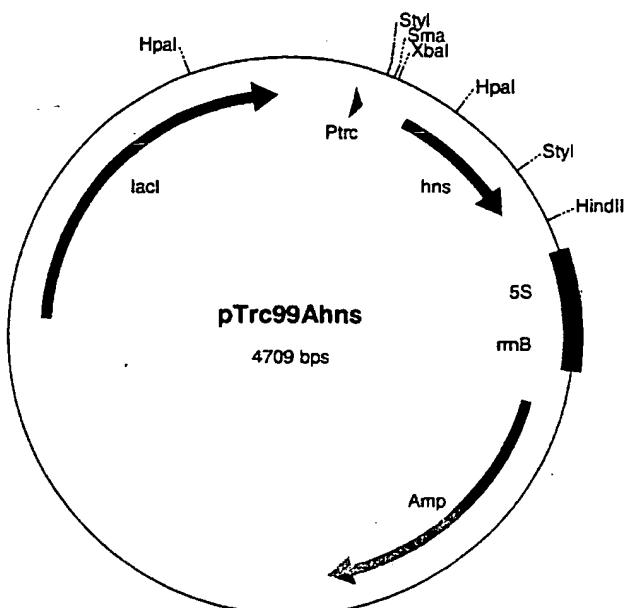
(75) Inventor/Applicant (for US only): RIEPING, Mechthild  
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(88) Date of publication of the international search report:  
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY



WO 03/004671 A3

(57) Abstract: The invention relates to a process for the preparation of L-amino acids, in particular L-threonine.

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 02/06567

A. CLASSIFICATION OF SUBJECT MATTER					
IPC 7	C12P13/06	C12P13/08	C12P13/10	C12P13/12	C12P13/14
	C12P13/20	C12P13/22	C12P13/24		

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, MEDLINE, EPO-Internal, WPI Data, PAJ

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- \*&\* document member of the same patent family

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European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel: (+31-70) 340-2040, Tx. 31 651 epo nl.  
Fax: (+31-70) 340-3016

Authorized officer

Kools, P

## INTERNATIONAL SEARCH REPORT

International Application No

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E	WO 03 004664 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 ---	6
E	WO 03 004663 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 ---	6
E	WO 03 004575 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 ---	6
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International Application No	EP 02/06567
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E	WO 03 004670 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 ----	6
E	WO 03 004669 A (RIEPING MECHTHILD ;DEGUSSA (DE)) 16 January 2003 (2003-01-16) claim 6 ----	6
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International Application No

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